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Lipopolysaccharide-induced *MCP-1* gene expression in rat tubular epithelial cells is nuclear factor-κB dependent

YIPING WANG, GOPALA K. RANGAN, BRYAN GOODWIN, YUET-CHING TAY, YANG WANG, and DAVID C.H. HARRIS

Department of Renal Medicine and Storr Liver Unit, Department of Medicine, The University of Sydney at Westmead Hospital, Sydney, New South Wales, Australia

Lipopolysaccharide-induced *MCP-1* gene expression in rat tubular epithelial cells is nuclear factor-κB dependent.

Background. Endotoxin is an important factor in the development of acute renal failure related to infection and in acceleration of chronic nephritis. Lipopolysaccharide (LPS; the major component of endotoxin) is one of the most potent triggers for renal cells to produce monocyte chemoattractant protein-1 (MCP-1), a key cytokine involved in immune cell recruitment into the renal interstitium in acute and chronic renal diseases. Knowledge about the transcriptional regulation of *MCP-1* in renal tubular epithelial cells in response to LPS is incomplete.

Methods. Transcriptional regulation of *MCP-1* was investigated in rat proximal tubule cells (PTCs) in primary culture and was exposed to LPS using electromobility shift assay and supershift analysis for nuclear factor-κB (NF-κB) and Western blot for the NF-κB inhibitory protein IκB. To prove the role for NF-κB, activator protein (AP-1), and sequence-specific transcription factor (Sp1), mutation and deletion analysis was performed using a 3.5 kb fragment of rat MCP-1 5′-flanking region inserted into a luciferase reporter construct transfected into tubular epithelial cell line (NRK-52E).

Results. LPS increased NF-κB in a dose- and time-dependent manner, which paralleled that of MCP-1 mRNA expression. IκBα decreased within 30 minutes of LPS treatment, but returned to basal levels by two hours. IκBβ levels were depressed within one hour and remained low throughout the culture period after LPS stimulation. The activity of the transfected 5′-flanking region of the *MCP-1* gene increased nearly threefold after LPS stimulation. Mutation or deletion of NF-κB binding sites, located in the enhancer region of the 5′-flanking region, resulted in a total loss of LPS-induced increase in luciferase activity. Mutation of putative AP-1 and Sp1 sites, located in the proximal promoter region of *MCP-1*, reduced basal luciferase activity in unstimulated cells, but had no effect on LPS-stimulated luciferase activity.

Conclusions. These studies prove that NF-κB is critical for LPS-induced *MCP-1* transcription, and AP-1 and Sp1 are essential for basal expression of MCP-1 in rat tubule cells. The species-specific nature of transcriptional regulation of MCP-1 has important implications for the delineation of treatment to prevent endotoxin-mediated renal injury.

Key words: tubule cells, monocyte chemoattractant protein-1, NF-κB, renal injury, activator protein-1, sequence specific transcription factor.

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Lipopolysaccharide (LPS) is a product of gram-negative bacteria, which has been implicated directly in the pathogenesis of acute and chronic renal disease [1–4]. For example, LPS has been shown to accelerate certain types of experimental glomerulonephritis, including lupus nephritis, anti-glomerular basement membrane (anti-GBM) disease, and IgA nephropathy [2–4]. Moreover, LPS has recently been shown to cause proximal tubular cell injury [5]. LPS is a potent stimulus of cytokine production and has been shown to increase monocyte chemoattractant protein-1 (MCP-1) expression in mesangial cells [6] and renal cortical epithelial cells [7]. By its induction of MCP-1, it is possible that LPS could promote the interstitial inflammation, which characterizes both acute and chronic renal disease.

Interstitial inflammation is a constant pathologic feature of progressive chronic renal disease of all types and is a frequent finding in acute renal diseases as well [8, 9]. During the early phases of acute and chronic renal disease, macrophages form an important component of the inflammatory infiltrates within the interstitium [9]. However, the mechanisms underlying macrophage infiltration of the interstitium have not been fully elucidated. Of the various cytokines that are produced locally, the monocyte-specific CC chemokine MCP-1 appears to play a predominant role in the regulation of interstitial inflammation. Proximal tubule cells (PTCs) have been shown to be an important source of MCP-1 in both human and experimental chronic renal diseases [10, 11].

The induction of MCP-1 following exposure to proteins and other urinary components may be important in the development of interstitial inflammation in chronic glomerular diseases. In a previous study, we showed that the transcription of MCP-1 in PTCs stimulated by albu-

min correlated with activation of the transcription factor nuclear factor-κB (NF-κB). We further demonstrated that the 5′-flanking region of rat *MCP-1* gene (which exhibited a 64% homology with the human MCP-1 5′-flanking region) contained binding sites for NF-κB, activator protein (AP-1), and sequence-specific transcription factor (Sp1) [12].

A number of studies have focused on the regulators of murine and human *MCP-1* genes [13, 14]. However, much less is known about the regulation of MCP-1 in rats, the species frequently used in in vivo studies of acute and chronic renal disease. As LPS is likely to be a clinically important stimulus for MCP-1 production in a variety of animal and human renal diseases, this study sought to investigate the transcriptional regulation of rat MCP-1 in PTCs in response to stimulation with LPS. We hypothesized that the signal transduction pathways of *MCP-1* gene in PTCs require the activation of NF-κB, AP-1, and Sp1. To investigate this hypothesis, the role of these factors in MCP-1 regulation was examined by transient transfection of chimeric MCP-1-luciferase reporter gene constructs containing MCP-1–regulatory regions into a rat tubular epithelial cell line.

METHODS

Reagents

Dulbecco's modified Eagle's medium (DMEM), LPS (*Escherichia coli*, serotype 026:B6), dexamethasone, N-tosyl-phe-chloro-methyl-ketone (TPCK), and all other cell culture reagents were purchased from Sigma-Aldrich (Sydney, Australia). Plasmid pGL3-basic and luciferase assay system, gel shift assay system, and oligonucleotide labeling kit were from Promega (Sydney, Australia). The rabbit polyclonal antibodies against p50, p65, c-Rel, IκBα, and IκBβ were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). P^{32}-γATP, Hybond-C extra nylon, peroxidase-conjugated sheep antirabbit IgG, and enhanced chemiluminescence (ECL) detection reagent were from Amersham (Sydney, Australia). A transformer site-directed mutagenesis kit was from Clontech (Palo Alto, CA, USA), and transfection reagent lipofect AMIN-plus was from Life Technologies (Sydney, Australia). DNA-modifying enzymes, unless otherwise stated, were from Boehringer Mannheim (Sydney, Australia). The rat tubular cell line (NRK52E) was purchased from American Type culture Collection (Rockville, MD, USA).

Cells

For primary culture, PTCs were isolated and cultured from normal male Wistar rats using isopycnic centrifugation. Cells were grown in DMEM supplemented with epidermal growth factor (10 ng/mL) and insulin (5 μg/mL) in a 5% CO_2/95% O_2 environment [15, 16]. The proximal tubular cell origin of these cells was verified in previous experiments [16]. Experiments were commenced after cells had reached confluence, which was usually between five and six days after the isolation procedure. The NRK52E cell line was obtained originally from normal rat kidney at passage 15 and showed epithelial morphology. The cells were cultured in DMEM containing 5% fetal calf serum and were maintained at 37°C in 5% CO_2/95% O_2. The cells from primary culture or cell line were treated with or without LPS (0 to 10 ng/mL) for 0 to 16 hours in serum-free medium in various experiments.

Analysis of monocyte chemoattractant protein-1 transcription

Total RNA was isolated using RNAZOL B (Teltest, TX, USA). The transcription of MCP-1 mRNA in PTC was determined using semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR). The design of primers and conditions for RT-PCR have been described previously [12]. Ten percent of the PCR product was loaded into a 1.2% agarose gel stained by ethidium bromide (0.5 μg/mL) and was photographed (Polaroid 665 film) over ultraviolet light. The bands on the negative film were scanned by Densitometry (Personal Densitometer; Molecular Dynamics, Sunnyvale, CA, USA), and the signal intensity for MCP-1 was expressed relative to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*).

Extraction of nuclear protein and electrophoretic mobility shift assay

Nuclear extracts from PTCs were prepared using methods described previously [15, 17]. The nuclear proteins were diluted to a standard concentration (3 μg/μL) and were stored in aliquots at −70°C. Double-stranded oligonucleotides, containing NF-κB consensus binding sites, were radiolabeled using T4 polynucleotide kinase (Promega) and [^{32}P] ATP and were purified by centrifugation over a G-50 sephadex spin column. Specific oligonucleotide probes of NF-κB rat MCP-1-probe A (5′-GTCTGGGAACTTCCAATGC-3′, base −2287 to −2278) and MCP-1-probe B (5′-GAATGGGAATT TCCC-3′, base −2261 to −2252) carrying rat MCP-1 NF-κB consensus sequences and their mutations [mutation A (GTCTGGGAACTcggAATGC) and mutation B (GGAATGGccATTTCCACCAC)] were radiolabeled and employed to examine the specificity of NF-κB binding to the rat *MCP-1* gene. Five micrograms of nuclear protein were incubated with 50,000 cpm ^{32}P-labeled probes for 20 minutes at room temperature according to the manufacturer's instructions (Promega). The samples were loaded onto a 7% polyacrylamide gel with 1 × Tris-acetate/EDTA buffer, and electrophoresis was performed at 100 V for one hour. The gel was vacuum dried and exposed to x-ray film for two to four hours. The specificity of the reaction was determined by competition

reactions in which a 100-fold molar excess of unlabeled NF-κB probe was added to the binding reaction 10 minutes before the addition of a radiolabeled probe.

The composition of activated NF-κB was analyzed using a supershift assay. In these studies, 1 μL of antibodies reactive to the rat p50, p65, or cRel protein was incubated with the reaction mixture for 30 minutes and was then added to radiolabeled NF-κB probes (A and B) for 15 minutes. Electrophoresis was performed as described, and the autoradiographs were analyzed for reduction in signal intensity and the presence of a supershifted band.

Western blot analysis

Nuclear factor-κB activity is primarily regulated by the turnover of IκB proteins in the cytosol. Therefore, Western immunoblotting was performed to analyze the kinetics of IκB proteins. Cells were lyzed with buffer [0.1 mol/L Tris, pH 6.8, 0.1% Nonidet P-40, 10% β-mercaptoethanol, 4 mmol/L ethylenediaminetetraacetic acid (EDTA), and 1 mmol/L orthovanadate]. Protein content of the samples was determined. Samples containing 10 μg of protein were loaded into each lane and electrophoresed on a 10% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) under reducing conditions. The proteins were transferred to a nitrocellulose filter for electrophoresis performed for one hour at 100 V. To block nonspecific binding sites, the membrane was incubated sequentially with 5% bovine serum albumin and then primary polyclonal antibodies (rabbit anti-IκBα and anti-IκBβ, 1:1000) for one hour each at room temperature. Blots were washed with Tris buffer and incubated with peroxidase-conjugated sheep antirabbit IgG (1:1000) for one hour at room temperature. Reactivity was detected with ECL detection reagent. The molecular weights of the immunoreactive bands were estimated by comparison with known molecular standards.

Construction of plasmids

Previously, the *rat MCP-1* (*rMCP-1*) gene was cloned and sequenced up to −940 bp upstream, which does not encompass all of the regulatory regions delineated in this study [18]. To elucidate the mechanism of *rMCP-1* transcriptional regulation, the 5′-flanking region of the *rMCP-1* from base −3580 to +80 was cloned and sequenced [15]. The relevant sequence of the 5′-flanking region of the *rMCP-1* gene is illustrated in Figure 1. Two NF-κB binding sites were found in the counterpart of human *MCP-1* enhancer region: A, 2287/−2278, and B, 2261/−2252, relative to the major transcriptional start site. In the proximal promoter region of *rMCP-1* gene, an AP-1 binding site at base −54 to −44 and Sp1 binding site at base −51 to −39 were identified. The 5′-flanking region of *rMCP-1* from base −3555 to +74 was amplified from rat genomic DNA by PCR using the sense primer, 5′-CTCAAAGGTGCTGCAGAGTTACTT-3′, and antisense primer, 5′-TGCATAGTGGTGGAGGAAGA-3′. The resultant amplicon was gel purified (Qiagen Pty Ltd., Victoria, Australia), digested with *Bgl* II, and inserted into *Bgl* II-linearized pGL3-basic to form pGL3-MCP-1. The 215 bp *rMCP-1* promoter region between −107 and +74 was amplified by standard PCR using the sense primer 5′-AGCAGATTCAAACTTCCA-3′ and antisense primer 5′-GGAGGAAGAGAGATTCGAAGG-3′ and was digested with *Hind* III and *Xho* I and inserted into *Hind* III-*Xho* I–linearized pGL3-basic to form pGL3-MCP (−107/+74). The 343 bp *rMCP-1* enhancer region between −2407 and −2065 was amplified using a sense primer 5′-GAGCTCAGACTATGCCTTTGT-3′ and antisense primer 5′-GAGCCTGGGAGGTCACCATT-3′. The resultant fragment was digested with *Kpn*I and *Xho* I and inserted into *Kpn*I-*Xho* I-linearized pGL3-TK, which contains bases −105 to +53 of the herpes simplex virus thymidine kinase (TK) promoter linked to a luciferase reporter gene to form pGL3-MCP (−2407/−2065).

Mutagenesis

Site-directed mutagenesis was performed on the putative NF-κB binding sites in the distal enhancer region using the Transformer Site-Directed Mutagenesis kit according to the manufacturer's instructions. The oligonucleotides used were 5′-GGAATGGccATTTCCACC AC-3′ and 5′-GTCTGGGAACTcggAATGC-3′ to obtain mutated NF-κB construct pGL3-κB-MA and pGL3-κB-MB. To delete the NF-κB binding sites A and B, the pGL3-MCP (−2219/+74) was prepared by excising a fragment from −2219 upstream on pGL3-MCP-1. The putative Sp1 and AP-1 binding sites in the proximal promoter region were revealed by nucleotide sequence analysis and mutated using the oligonucleotides 5′-GCCTGACTCCAagCTCTGGCTTAC-3′ and 5′-CAC CCTGCCTGgtaCCACCCTCTG-3′ to form the mutated Sp1 and AP-1 construct pGL3-SP-M and pGL3-AP-M.

Cell transfection and luciferase assay

In six-well cell culture plates, NRK-52E cells were seeded at a density of 3×10^5 cells per well in 1 mL medium. After 20 hours of incubation, the cells were 60 to 80% confluent. The NRK52E cells were cotransfected with luciferase expression vector (6 μg/mL) and β-gal vector (3 μg/mL as control) by lipofectAMIN-plus following the instructions of the kit. After 24 hours of transfection, the cells were incubated with or without LPS for eight hours and then harvested. The luciferase activity in cell extracts was determined by an α-counter following the instructions of luciferase assay kit, and β-gal activity was assayed by spectrophotometer.

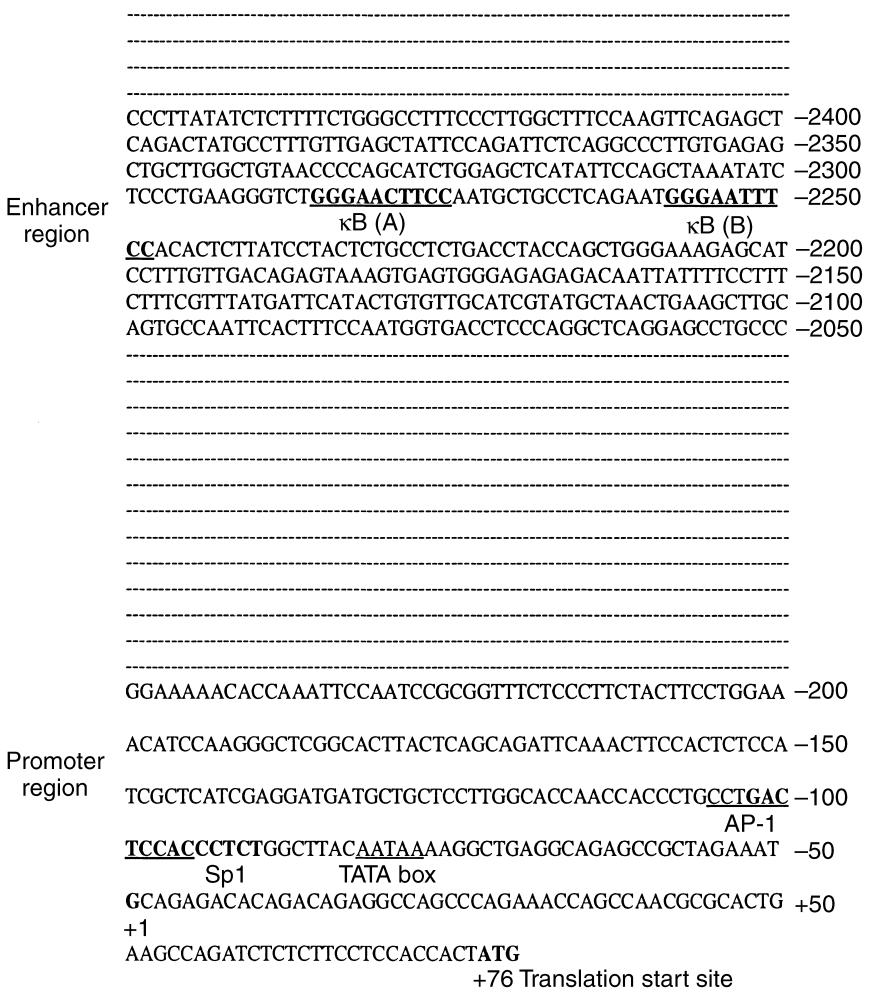

Fig. 1. Sequence of the enhancer and promoter region of rat monocyte chemoattractant protein-1 (*rMCP-1*) gene. The κB binding sites A (−2287/−2278) and B (−2261/−2252) in the enhancer region are underlined. The activated protein-1 (AP-1) binding site (−54/−44) is underlined, and the sequence specific transcription factor (Sp1) binding site (−51/−39) is highlighted in promoter region.

Statistics

Data are presented as mean ± SD of three to five separate experiments in which values were determined in triplicate. Analysis of variance (ANOVA) and Fisher's least significant method were used for comparisons among multiple means, and the Student's unpaired *t*-test was used for comparison between two means. A *P* value less than 0.05 was considered significant.

RESULTS

Nuclear factor-κB activation and monocyte chemoattractant protein-1 mRNA expression

The activation of NF-κB in response to LPS was examined using an NF-κB consensus binding oligonucleotide. Two bands, upper band (complex I) and lower band (complex II), appeared on the electrophoretic mobility shift assay (EMSA; Fig. 2). Nuclear protein incubated with labeled NF-κB oligonucleotide and 1 to 10 times the molar excess of unlabeled oligonucleotide reduced the density of complex I in a dose-dependent manner, but had no effect on the density of complex II. Incubation with labeled NF-κB and unlabeled mutant NF-κB probe (1 to 10×) only reduced the density of complex II (data not shown). Therefore, complex II is likely to represent the binding by a non–NF-κB transcription factor, whereas complex I is specific for NF-κB binding. In unstimulated PTCs, there was weak binding activity of NF-κB. LPS stimulation (1 to 10 μg/mL) increased NF-κB binding activity dose dependently; maximal binding was observed at 5 μg/mL LPS (Fig. 2). NF-κB binding activity was strongly induced at 2 hours, peaked at 8 hours, and was maintained a high level at least 16 hours following stimulation with LPS. The addition of excess unlabeled NF-κB oligonucleotide completely abrogated complex formation, thereby confirming the specificity of the reaction (Fig. 3). The time- and dose-dependent activation of NF-κB paralleled that of MCP-1 mRNA accumulation. LPS (5 μg/mL) produce a fivefold increase in MCP-1 expression, with a peak at 4 to 8 hours and a sustained increase for up to 24 hours with continued exposure (Fig. 4). Pretreatment of cells with NF-κB in-

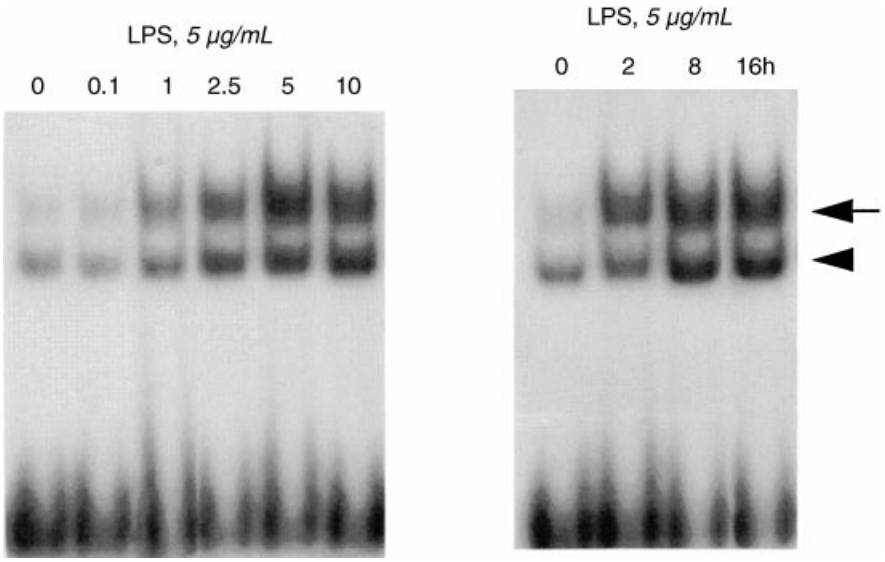

Fig. 2. Activation of nuclear factor-κB (NF-κB) by lipopolysaccharide (LPS) in proximal tubule cells (PTC). The arrow indicates the upper band (complex I), which is an NF-κB specific binding complex. The arrowhead indicates the lower band (complex II), which is a result of nonspecific binding. Electromobility shift assay (EMSA) shows the dose–response pattern for NF-κB binding in nuclear extracts from proximal tubule cells stimulated by LPS (0 to 10 μg/mL) and the time course for NF-κB binding activity after exposure to LPS (5 μg/mL) for 0 to 16 hours.

hibitor TPCK resulted in an attenuated expression of NF-κB after exposure to LPS. In contrast, pretreatment of cells with dexamethasone (0.1 μmol/L) resulted in a partial suppression of NF-κB activity (Fig. 3). The dose response (0.01 to 10 μmol/L) was tested for dexamethasone; NF-κB activity was partially suppressed with as little as 0.03 μmol/L, and no extra suppression was seen for doses beyond 0.1 μmol/L. In parallel, LPS-induced expression of MCP-1 was inhibited by both TPCK and dexamethasone, as previously described [15].

Degradation of cytoplasm IκB

To further understand the mechanism of NF-κB expression in PTC stimulated with LPS, the levels of IκB-α and IκB-β were determined with Western blots of cytoplasmic extracts (Fig. 5). Stimulation of cells with LPS led to a rapid, but transient decrease in IκB-α. In cells stimulated by LPS, IκB-α was reduced within 30 minutes and returned to control levels within 4 hours, despite continued exposure. Levels of IκB-β also decreased, but unlike with IκBα, this was not seen until after a one-hour stimulation with LPS. Also unlike IκB-α, lower levels of IκB-β persisted under continuing stimulation with LPS.

Functional analysis of *MCP-1* gene regulation

An EMSA was performed to test whether NF-κB binds to the κB consensus sequences identified in the enhancer region of *MCP-1* gene in response to LPS. LPS increased the specific binding activity of NF-κB to probes containing the κB consensus sequences (probe A and probe B), but not to mutated probes (mutation A or mutation B; Fig. 6). Supershift analysis using specific probes that bind to κB consensus sites of *rMCP-1* showed that the complexes that formed on probe A and probe B contained the NF-κB components p50, p65, and c-Rel (Fig. 7).

To elucidate the mechanism of *rMCP-1* transcriptional regulation in tubular epithelial cells by LPS stimulation, the 3567 bp 5′-flanking region (base −3580 to +74) was cloned into a luciferase reporter plasmid. *rMCP-1* gene contains two NF-κB binding sites in an enhancer region that are identical to human MCP-1 (*hMCP-1*). However, unlike *hMCP-1*, there is no NF-κB binding site in the proximal promoter region of *rMCP-1.* As seen in Figure 8, tubule cells (NRK-52E cells) transfected with pGL3-MCP-1 demonstrated a significant increase in luciferase activity when exposed to LPS. The induced levels of luciferase activity were twofold to threefold greater than in unstimulated cells. To investigate which regions are involved in the transcriptional activity of *rMCP-1* by LPS, tubule cells were transfected with pGL3-MCP (−2407/−2065), which contains the enhancer region of MCP-1 linked to a minimal TK promoter, and exhibited an fivefold increase of luciferase activity in response to LPS. However, tubular epithelial cells transfected with pGL3MCP (−107/+74), which contains the MCP-1 promoter region alone, showed no change in luciferase activity when incubated with LPS. This indicated that the sequence of promoter region was not essential for induction of MCP-1 by LPS.

To examine the role of NF-κB, AP-1, and Sp1, tubular epithelial cells were transfected with plasmids containing mutations in these binding sites. Mutants of the natural NF-κB binding sites were generated and analyzed by transfection in tubule cells (Fig. 9). Mutagenesis of NF-κB binding site A elicited a significant reduction in luciferase activity in both unstimulated controls and cells exposed to LPS, although luciferase activity was still induced twofold in LPS-stimulated versus control cells.

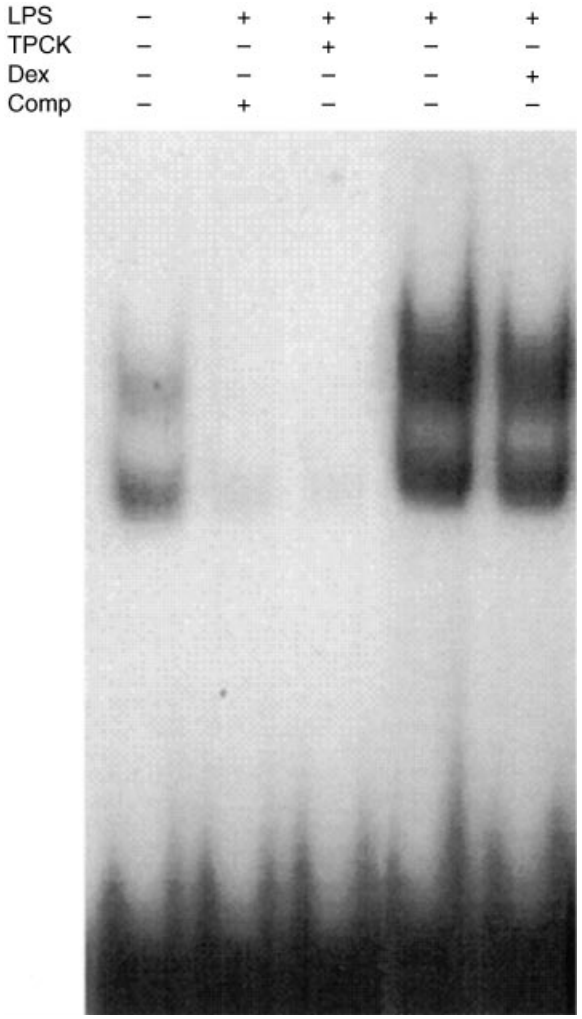

Fig. 3. Inhibition of NF-κB. EMSA shows that NF-κB binding activity after stimulation by LPS (5 μg/mL) was completely inhibited by TPCK (100 mmol/L) and excess unlabeled NF-κB oligonucleotide (Comp) and partially inhibited by dexamethasone (Dex. 0.1 μmol/L). This is representative of five separate experiments.

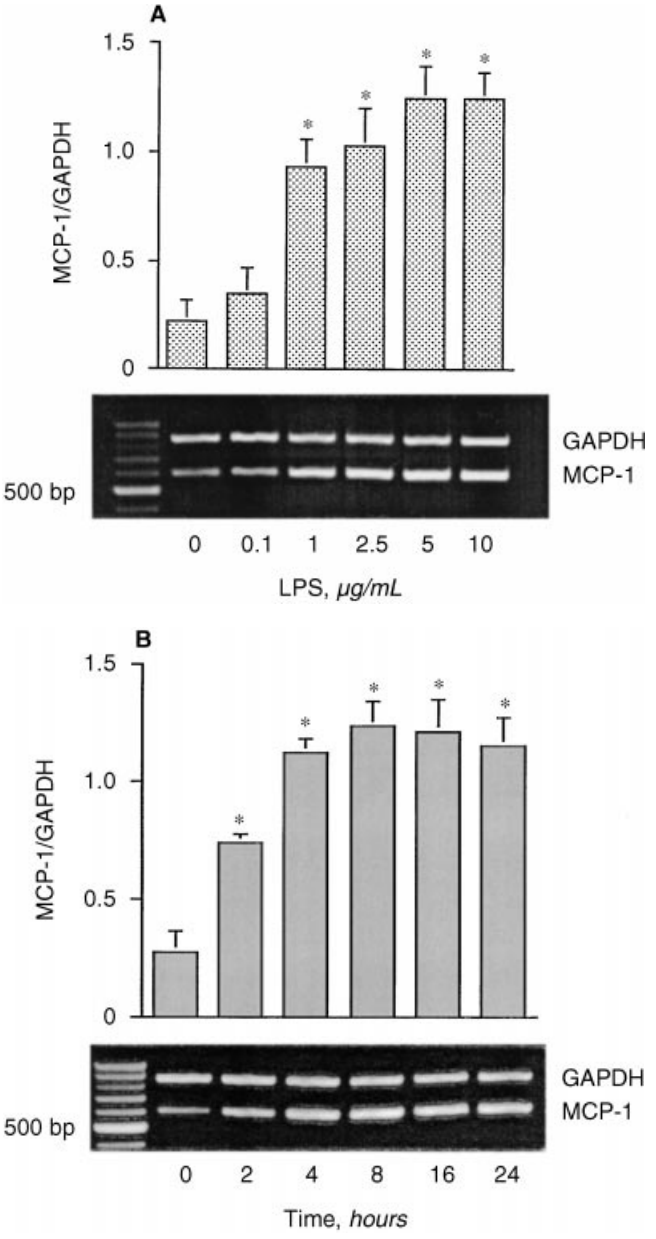

Fig. 4. Expression of MCP-1 mRNA relative to GAPDH by reverse transcriptase polymerase chain reaction (RT-PCR) in PTC stimulated by LPS. (*A*) PTCs were exposed to LPS in concentrations up to 10 μg/mL for eight hours. (*B*) PTCs were exposed to 5 μg/mL LPS for 0, 2, 4, 8, 16, and 24 hours. $*P < 0.01$.

In contrast, mutation of NF-κB binding site B resulted in complete loss of LPS-induced luciferase activity. This indicated that NF-κB binding site B is critical to the regulation of *rMCP-1* transcription by LPS. Following the deletion of both NF-κB binding sites A and B in the enhancer region, the basal reporter gene activity was dramatically reduced, and LPS effects were abolished. This demonstrated that the NF-κB binding sites in the distal enhancer region were necessary for LPS to induce *rMCP-1* transcription.

We next examined the effect of mutation of AP-1 and Sp1 sites in the promoter region of *rMCP-1* (Fig. 10). A mutation of the AP-1 site substantially reduced reporter gene expression to approximately 60% of the wild-type activity. Likewise, mutation of the putative Sp1 binding motif was shown to reduce reporter gene activity to 30% of wild-type expression. These results suggest that AP-1 and Sp1 play an important role in maintaining the basal transcriptional expression of *rMCP-1*. Neither AP-1 nor Sp1 in the promoter region increased luciferasis activity under stimulation of LPS. Moreover, LPS stimulation caused no effect on luciferase activity induced by either AP-1 or Sp1 mutants. This indicated that LPS-induced *rMCP-1* transcription is not dependent on either AP-1 or Sp1 binding sites in the promoter region.

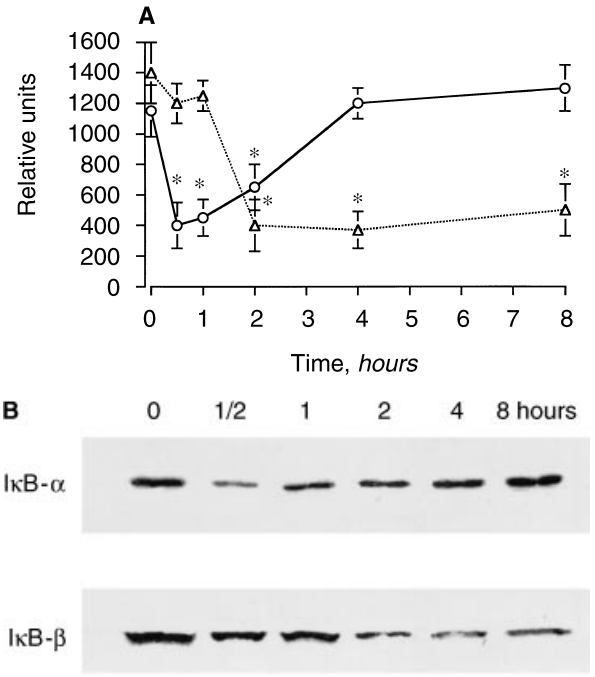

Fig. 5. Expression of IκBα (○) and IκBβ (△) in PTCs stimulated by LPS (5 μg/mL). (*A*) IκBα levels decreased rapidly and returned to baseline after four hours of stimulation, whereas IκBβ levels decreased after one hour and did not recover by eight hours ($N = 3$). $^*P < 0.001$ vs. baseline. (*B*) Representative Western blots showing the changes in IκBα (upper panel) and IκBβ (lower panel) levels in cytoplasmic extracts of PTCs exposed to LPS.

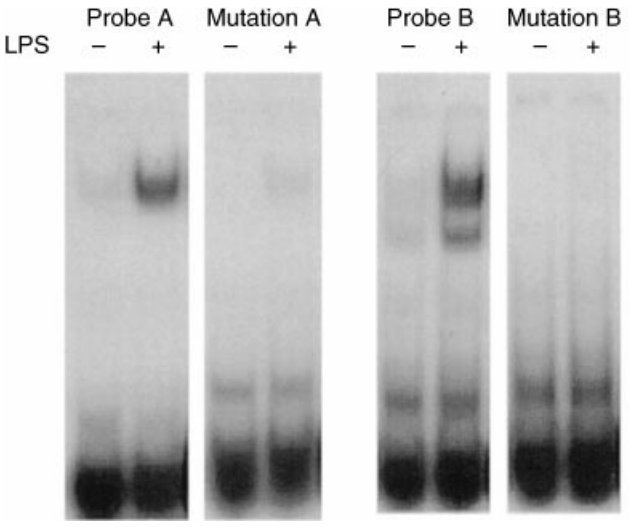

Fig. 6. NF-κB binding to κB sites of rat MCP-1 gene. Exposure to LPS (5 μg/mL) for eight hours increased NF-κB binding activity to probe A and probe B containing NF-κB consensus sequences, but not their mutations, mutation A and B. This is representative of three separate experiments.

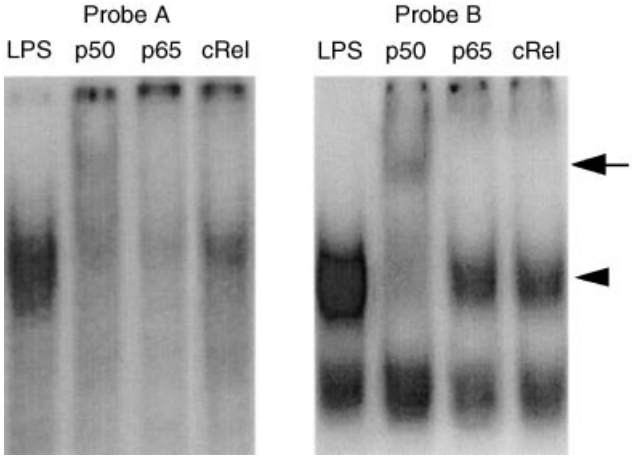

Fig. 7. Supershift analysis using specific probes that bind to κB consensus sites of rat MCP-1 revealed that activated NF-κB bands are composed of p50, p65, and c-Rel. The lower arrowhead indicates NF-κB binding bands. The upper arrow indicates the supershifted band. The is representative of three separate experiments.

DISCUSSION

Despite intensive investigation of the *MCP-1* gene, there is little information known about the molecular mechanisms involved in transcriptional region of *MCP-1* in the rat species, as the 5′-flanking region of *rat MCP-1* gene was isolated and sequenced only recently. To our knowledge, the present study is the first direct demonstration of transcription regulation of MCP-1 in rat renal tissue, using functional analysis of MCP-1 transcriptional regulatory region. In this study, we have shown that LPS activated NF-κB in a time- and dose-dependent manner, which correlated with MCP-1 expression. Furthermore, inhibition of NF-κB with TPCK or dexamethasone fully or partially reduced MCP-1 expression. Moreover, mutation or deletion of NF-κB binding sites in the enhancer region attenuated LPS-induced transcription activity and reduced basal transcription activity of reporter gene constructs. However, mutation of AP-1 or Sp1 binding sites in the promoter region only reduced basal transcription activity of reporter gene. These results demonstrate that the induction of MCP-1 transcription in PTCs by LPS depends on the activation of NF-κB, but not transcription factors AP-1 or Sp1.

Three functional NF-κB binding sites have been demonstrated to be important for the transcription of both human and murine *MCP-1* gene: one in the proximal promoter region and two in the distal enhancer region [19]. Interestingly, unlike the *hMCP-1* gene, *rMCP-1* has no NF-κB binding site in its promoter region. However, two NF-κB binding sites exist in the enhancer region of *rMCP-1* gene, and these are identical to *hMCP-1.* According to studies on *hMCP-1* gene, the κB site B (5′-GGGAATTTCC) sequence is identical to inter-

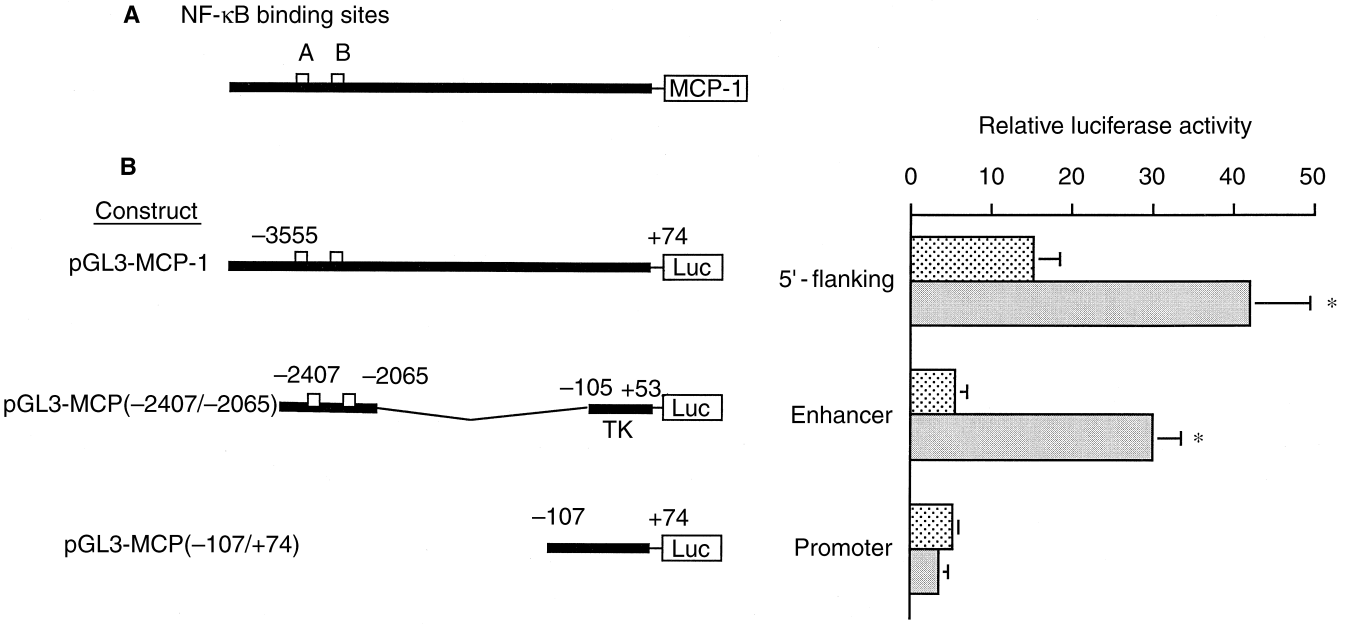

Fig. 8. Transactivity of different fragments of 5′-flanking region of *rMCP-1* gene in response to LPS stimulation. (*A*) Schematic structure of different regions of *rMCP*-1 gene. (*B*) Different fragments of 5′-flanking region linked to the luciferase gene were transfected into NRK-52E cells. The cells were stimulated with LPS (5 μg/mL) for eight hours, and the luciferase activity was measured. Values are means ± SD for at least three separate experiments and are normalized by cotransfected β-galactosidase activity. Symbols are: (▨) unstimulated; (■) LPS. The 5′-flanking region and enhancer region increased luciferase activity, whereas the promoter region had no effect on luciferase activity when incubated with LPS. *$P < 0.01$ vs. unstimulated cells.

feron-beta (INF-β), tumor necrosis factor-α (TNF-α) and multiple histocompatibility complex (MHC) class II associate invariant chain κB sequence [13, 20]. The κB site A (5′-GGGAACTCC) with sequence similarity to site B has a high affinity for both p65 and c-Rel, as distinct from the NF-κB binding site on *IL-8* gene, which has a high affinity for only p65, and on *Ig κ*-chain gene, which has a high affinity to only p50 [21, 22]. An interesting finding in this study is that both NF-κB binding sites have a high affinity not only for p65 and c-Rel, but also for p50, suggesting these binding sites in the *rMCP-1* gene have broader binding affinities for different NF-κB/Rel proteins than do the sites in human *MCP-1* gene. Of more importance to gene regulation than binding is the *trans*activating potential of p50, p60 and cRel, which is not clear. For example, Sheppard et al have shown that although NF-κB sites within the vascular cellular adhesion molecule-1 promoter region bind p50/p50 and p65/p65 homodimers and p50/p65 heterodimers, only p50/p65 heterodimers lead to gene activation during neural differentiation [23].

To further confirm the role of NF-κB in MCP-1 production, the κB binding sites (sites A and B) in the enhancer region were mutated, resulting in a loss of NF-κB binding ability and a reduction of gene transcription. Mutation of site A resulted in a partial loss of gene transcription in response to LPS, whereas mutation of site B caused a complete loss of LPS-induced transcription. This demonstrated that NF-κB binding site B plays the dominant role in LPS-mediated transcription of MCP-1 expression in rat tubule cells.

A LPS-induced increase of *hMCP-1* gene transcription depends on the activity of its enhancer and/or promoter region [14]. In the present studies, distal enhancer regions were capable of conferring LPS responsiveness on luciferase reporter gene, whereas the proximal promoter region of *rMCP-1* was not LPS responsive. This suggests that in rat, unlike humans, the enhancer region has the predominant role in LPS-induced MCP-1 transcription.

Monocyte chemoattractant protein-1 gene expression is stimulus and cell specific [24]. This observation is explained mainly by specific differences in the process of NF-κB activation. The mechanism by which LPS activates NF-κB in PTCs is not clear, but is probably mediated by phosphorylation of IκB. IκB is a family of inhibitors that complex with NF-κB in the cytoplasm and tightly control the nuclear translocation of NF-κB. Among the different IκB, IκBα and IκBβ play a major role in regulation of NF-κB [13, 25]. Studies have concluded that TNF-α or phorbol 12-myristate 13-acetate (PMA) induce degradation of IκBα but not IκBβ, suggesting that these IκB proteins are regulated separately in a stimulus-dependent manner [26, 27]. In the current study, increased nuclear binding of NF-κB was accompa-

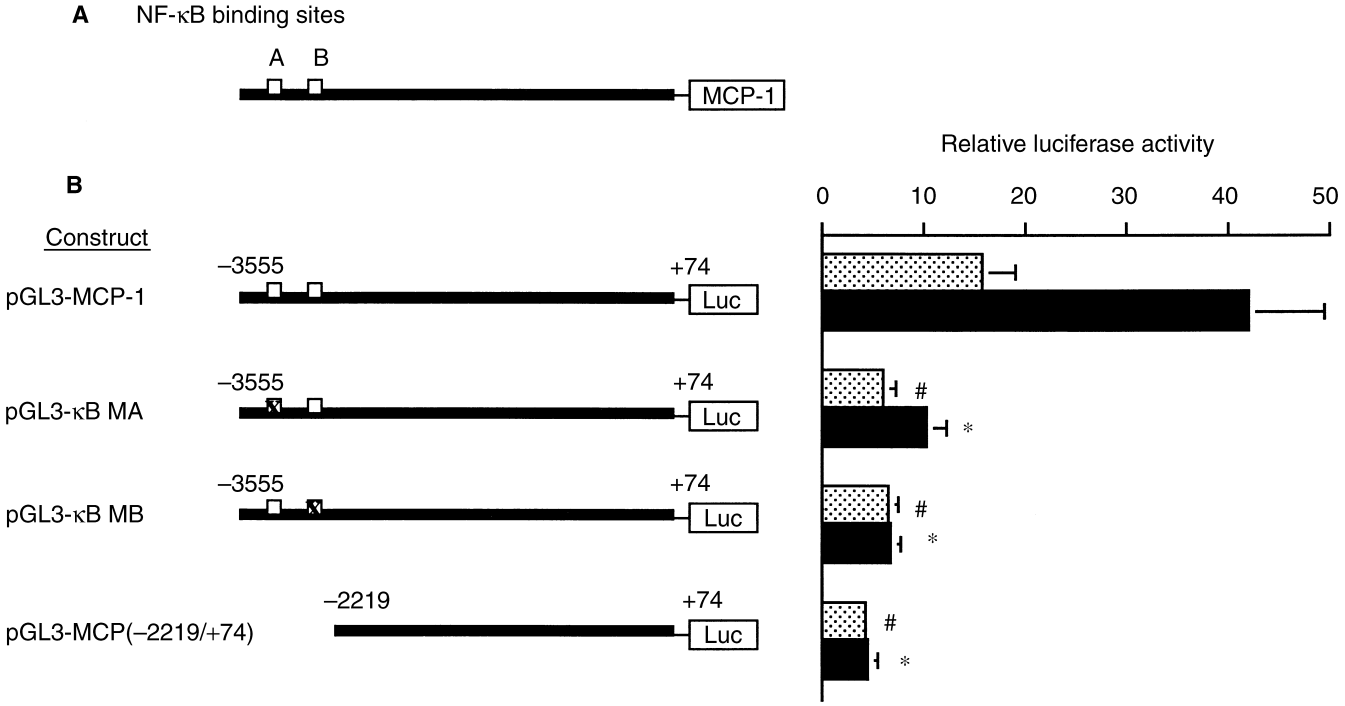

Fig. 9. Mutation and deletion analysis of the distal κB sites in enhancer region regulating MCP-1 induction. (*A*) Schematic structure of 5′-flanking region of the MCP-1 gene, indicating the two κB binding sites A and B. (*B*) κB sites in the enhancer region were mutated or deleted, and the resultant gene fragment was linked to the luciferase gene and transfected into NRK-52E cells. The cells were stimulated with LPS (5 μg/mL) for eight hours, and the luciferase activity was measured. Values are means ± SD for at least three separate experiments and are normalized by cotransfected β-galactosidase activity. κB sites A and B are shown by squares and mutated sites are marked with an *x*. Symbols are: (▩) unstimulated; (■) LPS. #$P < 0.01$ vs. unstimulated cells transfected with unmutated 5′-flanking region. *$P < 0.01$ vs. stimulated cells transfected with unmutated 5′-flanking region.

nied by a decline in IκB concentration. LPS led to a rapid and transient reduction in IκBα levels, but a later and persistent decrease in IκBβ. The reappearance of IκBα has been proposed to be an internal counter-regulatory mechanism to prevent excessive activation of NF-κB [28]. From this study, it appears that up-regulation of MCP-1 by LPS is caused by activation of NF-κB via temporary degradation of IκBα and persistent degradation of IκBβ. The rapid degradation of IκBα may contribute to early activation of NF-κB, and persistent degradation of IκBβ contributes to more prolonged activation of NF-κB.

Several studies have shown that pathways of *MCP-1* gene activation involve multiple transcription factors, including Sp1 and AP-1. Nucleotide sequence analysis of *rMCP-1* gene revealed a potential Sp1 binding motif. Previously, Ueda et al demonstrated that this site was important for the basal expression of *hMCP-1* gene [14]. However, the Sp1 binding site in *rMCP-1* is not identical to that of *hMCP-1*, and it was unclear whether this site in the rat gene was involved in basal expression. Indeed, in the present study, a mutation of the Sp1 site in *rMCP-1* substantially reduced the transcriptional activity of reporter gene constructs, demonstrating that this, in line with observations made with *hMCP-1*, is a functional *cis*-acting element.

Activated protein-1 is another important regulatory element involved in the regulation of many cytokine genes, including *MCP-1*. It has been reported that mutation of either AP-1 or NF-κB binding sites in the promoter region suppressed cytokine-induced MCP-1 expression in human endothelial cells, suggesting that these two elements act in a cooperative manner to regulate MCP-1 production [29]. However, in this study, mutation of the AP-1 site reduced only the basal transcription level of *rMCP-1*, but had no effect on LPS-induced MCP-1 expression. The explanation for this observation may be related to the lack of NF-κB binding sites in the promoter region of rat *MCP-1* gene or the possibility that AP-1 does not play a major role in rMCP-1 induction by LPS.

It has been proposed that inhibition of NF-κB might be useful therapeutically to alter the progression of chronic inflammation. Although glucocorticoids are capable of inhibiting NF-κB binding activity by LPS, interleukin-1 (IL-1), and TNF-α in lymphocytes and endothelial cells, a recent study showed that they were unable to do this in mesangial cells [30]. In our study, activation of NF-κB and induction of MCP-1 by LPS were completely blocked

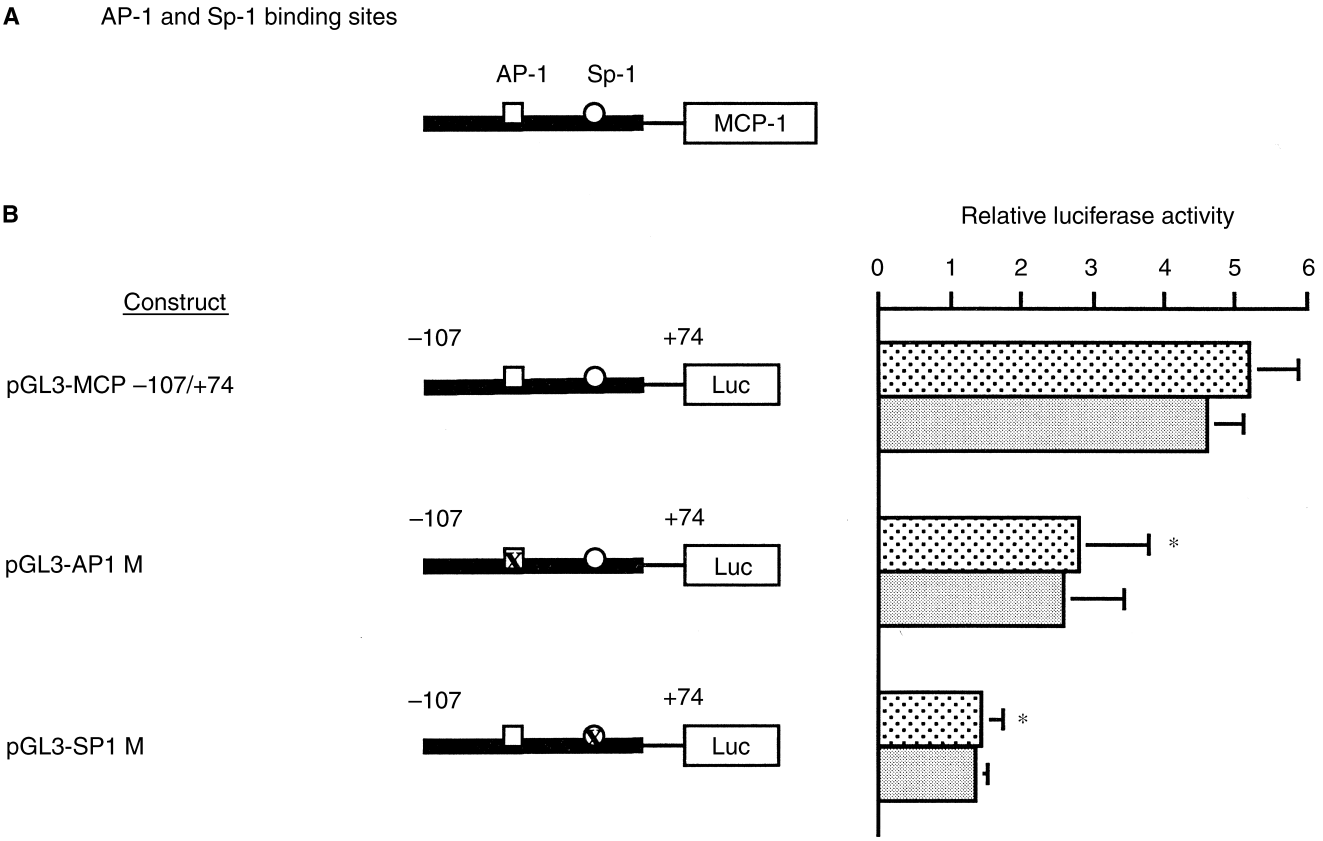

Fig. 10. Mutation analysis of AP-1 and Sp1 sites in the promoter region of the *rMCP-1* gene. (*A*) Schematic structure of promoter region of the *MCP-1* gene, showing the AP-1 and Sp1 binding sites. (*B*) AP-1 or Sp1 sites in promoter region were mutated, and resultant fragments were linked to the luciferase gene and transfected into NRK-52E cells. The cells were stimulated with LPS (5 μg/mL) for eight hours, and luciferase activity was measured. Values are means ± SD for at least three separate experiments and are normalized by cotransfected β-galactosidase activity. The AP-1 site is represented by a square and the Sp1 site by a circle, and mutated sites are marked with an *x*. Symbols are: (▩) unstimulated; (▨) LPS. $*P < 0.01$ vs. unstimulated cells transfected with intact promoter region.

by TPCK (an inhibitor of cytoplasmic IκB proteolysis) but only partially suppressed by dexamethasone. The reason for partial efficacy of steroids in this situation remains uncertain, but may be explained by cell specificity of response. It has been demonstrated that up-regulation of IκBα by glucocorticoids is not sufficient to block NF-κB activation in endothelial cells and epithelial cells [31, 32].

Lipopolysaccharide is a constituent of various gram-negative bacteria that are present in the gut in substantial amounts and a common resource of subclinical and clinical infection. The mechanism of action of LPS is related to CD14, a membrane protein that is considered to be a receptor for the LPS complex [33]. In animal models, LPS has been shown to exacerbate the severity of renal damage in lupus nephritis, anti-GBM disease and IgA nephropathy [2–4]. Furthermore, subclinical and clinical infections could accelerate chronic renal disease. The mechanisms by which LPS accelerate renal disease are unknown and have been proposed to involve activation of B cells to produce large amounts of immunoglobin and immune complexes [34]. However, Cavallo and Granholm found that activation of proliferative lupus nephritis by LPS could not be attributed to altered concentration of complement nor to delayed removal of circulating pathogenic immune complexes [35]. Such observations suggest the presence of alternative explanations for promotion of nephritis by LPS. Endothelial cells are generally thought to be the primary target of LPS, but recently, tubular epithelial cells have also been demonstrated to be important early targets of LPS [5]. Trachtman et al have shown that interactions between renal tubular epithelial cells and *E. coli* can stimulate nitric oxide production in renal cells and suggested that nitric oxide production could responsible for tubulointerstitial inflammation [36]. In the present studies, LPS directly activated NF-κB and induced MCP-1 transcription in PTCs. It is possible that such a mechanism might underlie the acceleration of nephritis by LPS, that is,

induction of MCP-1 leading to infiltration of leukocytes into the interstitium.

In summary, LPS induced MCP-1 expression in a time- and dose-dependent manner, which correlated with NF-κB activation and was inhibited fully or partially with NF-κB inhibitors TPCK or dexamethasone. Deletion or mutation of the NF-κB binding sites in the enhancer region of *rat MCP-1* gene dramatically reduced transcription activity in response to LPS, whereas mutation of AP-1 and Sp1 binding sites in the promoter region resulted in reduced basal transcription level of *rat MCP-1* gene. In conclusion, these studies prove that the up-regulation of MCP-1 transcription in rat tubule cells by LPS depends on NF-κB activation. This information could be useful in the design of anti-inflammatory strategies to specifically suppress transcriptional activation of MCP-1 in rat models of renal disease.

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Reprint requests to David C.H. Harris, M.D., Department of Renal Medicine, Westmead Hospital, Westmead NSW 2145, Australia.
E-mail: dch@renal.wh.usyd.edu.au

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